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Influence of Aae Autotransporter Protein on Adhesion and Biofilm Formation by *Aggregatibacter actinomycetemcomitans*

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The periodontopathogen *Aggregatibacter actinomycetemcomitans* colonizes oral cavity by binding to and invading epithelial cells as well as by participating in biofilms formed on hard surfaces. Aae, an autotransporter protein, is implicated in bacterial adhesion to epithelial cells. Due to the multiple functions of bacterial autotransporter proteins, this study aimed to evaluate the role of aae in *A. actinomycetemcomitans* ability to adhere to both saliva-coated hydroxyapatite (SHA) and biofilm. An *aae* null mutant was constructed. Its hydrophobic properties as well as its ability to adhere to epithelial cells, SHA and to form biofilm were evaluated and compared with the parental strain, *A. actinomycetemcomitans* VT1169. The aae null mutant showed reduced hydrophobicity, as well as decreased binding to SHA and biofilm formation compared to the parental strain. These data suggest that aae mediates *A. actinomycetemcomitans* adhesion to epithelial cells and may be involved in biofilm formation and interaction with adsorbed salivary proteins.

Key Words: aae, *Aggregatibacter actinomycetemcomitans*, adhesion, biofilm formation, hydrophobicity.

Introduction

Adhesion of bacteria to host tissues is the first step in the infectious process (1). The expression of bacterial outer membrane proteins is influenced by both environmental (2) and host factors and has specific interactions with host complementary receptors (3,4). Thus, changes in these bacterial outer membrane proteins may lead to alterations in hydrophobic properties and result in reduced attachment of bacteria to immobilized salivary proteins adsorbed to tooth surfaces or oral mucosa (5).

Adhesive type IV pili mediate *Aggregatibacter actinomycetemcomitans* ability to colonize tooth surface (1). However, ability to adhere to saliva-coated hydroxyapatite (SHA) is decreased and requires interaction with salivary proteins (6). Non-fimbriated variants exhibiting smooth colony morphology may still adhere to epithelial cells and form biofilms mediated by non-fimbrial components (7-10).

Three autotransporter proteins were recognized as having a role in the interaction of *A. actinomycetemcomitans* with the host: Aae, ApiA or Omp100 and EmaA (1,8-12). Autotransporter proteins are characterized by an amino-terminal leader peptide, a secreted mature protein (or passenger domain) and a C-terminal domain (13). These proteins present huge variations in passenger domain, explaining their wide range of effector functions, such as adhesion, invasion, biofilm formation, autoaggregation and cytotoxicity (14).

Aae and ApiA are both related to adhesion of *A. actinomycetemcomitans* to human buccal epithelial cells (10). An aae null mutant strain has shown decreased adhesion to KB cells, derived from oral carcinoma (8), and total loss of adhesion to buccal epithelial cells (BECs) was found in an aae/apiA double-null mutant (10). Aae specifically binds to BECs (11) and to blood vessel endothelial cells (HUVEC), but the domain involved in binding to BECs is located in a region of repeats, whereas a different domain is required for adhesion to HUVEC (15).

Aae is identical to the C-terminal region of *Haemophilus influenzae* IgA1 protease and Hap adhesin, but there is no significant homology of the N-terminal region with other autotransporter proteins listed in the GenBank (1) and this region is related to the BEC-binding domain (15). In Hap, the C-terminal 311 amino acid of the passenger domain (Hap_s) is involved in aggregation, microcolony formation and adhesion to epithelial cells. Furthermore, another domain, located in the C-terminal 511 residue of Hap is involved in binding to laminin, fibronectin and collagen IV (16).

Hence, the present study aimed to test the hypothesis that aae is also involved in A. *actinomycetemcomitans* ability to adhere to saliva-coated hydroxyapatite (SHA) and in biofilm formation. An aae null mutant was constructed and its ability to adhere to epithelial cells and to SHA as well as to form biofilms was evaluated and compared with the parental strain.

Material and Methods

Bacterial Strains, Plasmids and KB Cells

The bacterial strains used in the study are presented

in Table 1. *A. actinomycetemcomitans* strains were grown using Trypticase soy broth with 0.6% yeast extract (TSB-YE) in humidified 10% CO_2 incubator at 37 °C. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37 °C. For solid medium, 15 g/L of agar were added to liquid medium. Ampicilin, spectinomycin, kanamycin and rifampicin (100 µg.mL^{-1}) were added as needed. KB cells were kindly provided by Dr. Paula Fives-Taylor, University of Vermont. Cells were cultured in 75 cm^3 flasks with RPMI-1640 medium (Sigma Chemical Co, St. Louis, MO, USA) containing 5% of fetal bovine serum heat-inactivated (Cultilab, Campinas, SP, Brazil) and supplemented with 50 µg.mL^{-1} gentamycin at 37 °C in humidified atmosphere with 5% CO_2.

Aae Knock-Out Mutant

The aae gene was obtained by amplification using AAE5 (5'CAG AAC CAC AAC CAG TAC CAG CAC AC 3') and AAE3 (5'GCA GAA GTG AGT TAT TCA TCG 3') (24) primers and DNA from SUNY465 strain as template. PCR was performed in a Gene Amp PCR System 2400 (Perkin Elmer Inc., Norwalk, CT, USA) and consisted of one initial denaturation step (94 °C for 5 min) and 40 cycles of denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s and elongation at 72 °C for 2 min. The 2,732 bp fragment was cloned into pGEM-T Easy vector (Promega, Madison, WI, USA) and transformed in electrocompetent *E. coli* DH5α (p*aae*). Transformants were selected in LB supplemented with ampicilin. The *aae* gene was interrupted by insertion of a spectinomycin resistance gene obtained from pdl269. Plasmidial DNA was used as template to amplify the spectinomycin resistance gene, *aad*9, using forward primer (5' GGTACCAAAGCTTTTCGT TCG TGA 3') and reverse primer (5' CGCCATAAGCTTGGGTTATTGTT 3'). An initial denaturing step at 94 °C for 5 min was followed by 30 cycles at 94 °C for 1 min, 54 °C for 30 s and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. The 1,167 bp fragment was cloned into the pCR2.1-TOPO vector (Invitrogen Life Technologies, São Paulo, SP, Brazil) and transformed in *E. coli* TOPOF10. Transformants were selected in LB supplemented with kanamycin. Plasmid DNA (p*aad9*) was digested with *Hind*III. The 1,148 bp *Hind*III fragment of p*aad*9 was ligated to p*aae* and transformed in electrocompetent *E. coli* JM109. Transformants were selected in spectinomycin LB agar. The *aae*/*aad*9 fragment was obtained after double digestion with *EcoR*I and *Alw*21I (*AspH*I) (Fermentas Life Sciences, São Paulo, SP, Brazil) ligated into the conjugative plasmid pVT1460 (pVT*aae*/*aad*9) and transformed in competent *E. coli* SM10(λpir) cells. Transformant cells were selected in LB with spectinomycin and plasmid identity was confirmed.

An allelic replacement mutagenesis system was used (17) to generate the aae null mutant isogenic to *A. actinomycetemcomitans* VT1169 (SUNY 465, rifampicin and nalidixic acid resistant). *A. actinomycetemcomitans* VT1169, the recipient strain, and *E. coli* SM10(λpir)pVT*aae*/*aad*9 were grown in broth to reach $OD_{560\ nm}$ ~0.3 and ~0.5, respectively, and mixed. After conjugation, cells were grown on Trypticase soy agar with 0.6% yeast extract (TSA-YE) plates containing spectinomycin and rifampicin in 10% CO_2 at 37 °C for 48 h. Isolated colonies of putative transconjugants were screened by PCR using EXP5 (5' GCA TTT GCG TCA GAG TTT AAT G 3') and primers (8), located upstream and downstream *aa*e, respectively, exceeding

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Characteristics	Source (reference)
A. actinomycetemcomitans		
SUNY465	Clinical isolate, smooth phenotype, invasive; one copy of the *aae* repeat	Laboratory*
VT1169	SUNY465 Rif^R/Nal^R	Laboratory*
USP29	*aae* null mutant strain	This study
Escherichia coli		
JM109	Host for genetic constructions	Laboratory*
DH5α(lpir)	Competent cells	Laboratory*
SM10(lpir)	Conjugative competent cells	Laboratory*
TOPOF10	Electrocompetent cells used for cloning	Invitrogen
Plasmids		
pCR2.1-TOPO vector	Cloning PCR products (Kan^R)	Invitrogen
pGEM-T Easy vector sytem	Cloning PCR products (Amp^R)	Promega
pVT1460	Plasmid for allelic exchange	Laboratory*
pDL269	Spectinomycin resistance	Laboratory*
p*aad*9	Plasmid pCR2.1-TOPO + spectinomycin resistance gene	Laboratory*
p*aae*	Plasmid pGEM-T Easy+*aae*	This study
p*aae*/*aad*9	Plasmid pGEM-T Easy+*aae* disrupted with *aad9*	This study
pVT*aae*/*aad*9	Plasmid pVT1460+*aae* disrupted with *aad9*	This study

Laboratory*: Dr. Keith Mintz's and Dr. Paula Fives-Taylor's laboratory.

the cloned region of *aae* in pGEM-T Easy vector. Product identities were confirmed by sequencing, performed in MegaBACE100 with DYEnamic ET Dye Terminator Kit (GE Healthcare Bio-Sciences Pittsburgh, PA, USA).

Bacterial Cultures

A. actinomycetemcomitans VT1169 and USP29 (*aae*$^-$ mutant) were grown in TSB-YE added with rifampicin or spectinomycin until mid-exponential phase under microaerophilic (10% CO_2) incubation. The bacterial cultures were adjusted to an $OD_{500\ nm}$ of 0.20 corresponding to 3 x 10^8 cells.mL^{-1}.

Adhesion to KB Cells Assay

The binding capacity of the *aae* null mutant was determined by a standard adhesion assay (18). Approximately 2 x 10^5 KB cells/well were seeded in a 24-well tissue culture plate and incubated overnight in 5% CO_2 until confluence. Bacterial suspensions of *A. actinomycetemcomitans* parental strain (VT1169) and *aae* null mutant (USP29) were added to each well to reach a multiplicity of infection (MOI) of 1:100 (KB cell/bacteria). After 2 h of incubation at 37 °C, non-adhesive bacteria were removed by washing with PBS containing 1.0 mM $CaCl_2$/0.5 mM $MgCl_2$ and adherent/internalized bacteria were released with 0.1% Triton X-100. Samples were serially diluted and CFU estimated in TSA-YE plates. The experiment was performed in quadruplicate on three independent assays.

Adhesion to Hexadecane

The adhesion to hexadecane was assessed by a protocol modified as follows (5): bacterial cells were suspended in PUM buffer (9 mM K_2HPO_4, 50 mM KH_2PO_4, 30 mM urea, 0.8 mM $MgSO_4.7H_2O$, pH 7.1) to an $OD_{500\ nm}$ ~0.85 corresponding to 1 x 10^9 CFU mL^1, added with hexadecane (Sigma Aldrich, St. Louis, MO, USA). After phase separation, the fraction of adherent cells to hexadecane was estimated according the aqueous phase absorbance.

Biofilm Assay

Biofilm formation was estimated by a conventional crystal violet staining method (7). Aliquots of bacterial cultures at 10^6 CFU mL^{-1} were added to the wells of 96-wells flat-bottom polystyrene microtiter plates (Corning Inc., Corning, NY, USA) and incubated statically under microaerophilic atmosphere at 37 °C for 14 h. Total cell density was estimated by turbidity measurement at $OD_{490\ nm}$. After washing with phosphate-buffer saline (PBS, pH 7.3), adherent cells were fixed with methanol and stained with 0.1% crystal violet. The dye was eluted in 95% ethanol (v/v) and absorbance was measured at 600 nm. Biofilm formation was considered as the ratio between absorbance value of crystal violet eluted from biofilm ($OD_{600\ nm}$) and absorbance value obtained from total growth ($OD_{490\ nm}$). Experiments were made in sextuplicate. Non-inoculated wells were used as controls for sterility.

Adherence to Saliva-Coated Hydroxyapatite

The adherence to SHA was assessed by a protocol modified as follows (6): whole paraffin-stimulated saliva was obtained from six periodontally and medically healthy adults in wide mouth plastic tubes to achieve a minimum pooled volume of 40 mL. Salivary collection was divided into equal aliquots, frozen, clarified and heated to 60 °C for 30 min. Spherical hydroxyapatite beads (BDH Chemicals, Poole, U.K.) were added to the saliva and incubated for 2 h at 37 °C under slow rotation (5 rpm). The hydroxyapatite coated beads were washed, added with bacterial suspensions adjusted to ~3 x 10^8 CFU mL^{-1} in PBS and incubated for 2 h. After washing, cell/bead suspensions were subjected to sonication at low power impulse (Branson Ultrasonic Cleaner, Danbury, CT, USA) and allowed to settle down. Supernatants from non-sonicated (remnant non-adherent cells) and sonicated (adherent plus remnant non-adherent cells) tubes were inoculated on TSA-YE plates for CFU determination. The number of cells attached to SHA was the difference between CFU values determined in supernatant of sonicated and non-sonicated tubes. The experiment was performed in quadruplicate.

Statistical Analysis

Adhesion to KB cells, biofilm formation and adhesion to SHA of the parental and *aae*$^-$ mutant strains were compared by Student's t-test. Adhesion to n-hexadecane was evaluated by two analyses of variance followed by multiple comparisons Tukey's test. The significance level was set at $p<0.05$.

Results

Construction of an aae Knock-Out Strain

The chromosomal *aae* gene from VT1169 was interrupted by insertion of a spectinomycin cassette (*aad*9) in the *Hind*III unique site of *aae* by allelic replacement mutagenesis. Insertion was confirmed by amplification (. 1) using primers located upstream and downstream the cloned *aae* gene (EXP5 and AAE3) and sequencing. No differences in growth curves were observed between parental (VT1169) and its isogenic mutant *aae*$^-$ strains (USP29) (data not shown). Both strains reached the mid exponential growth phase after 6 h in microaerophilic incubation.

Adhesion to KB Cells

As expected, the *aae*$^-$ mutant exhibited a marked

reduction in adhesion to KB cells compared with the parental strain ($p<0.01$, Student's t-test). Results were reproducible in three independent assays (Fig. 2A).

Adherence to Hexadecane

Bacterial cells' hydrophobic properties differed between parental and *aae* null mutant strains, with *aae*⁻ mutant cells being more hydrophilic than those of the parental strain (Fig. 2B) ($p<0.0001$, Tukey's test).

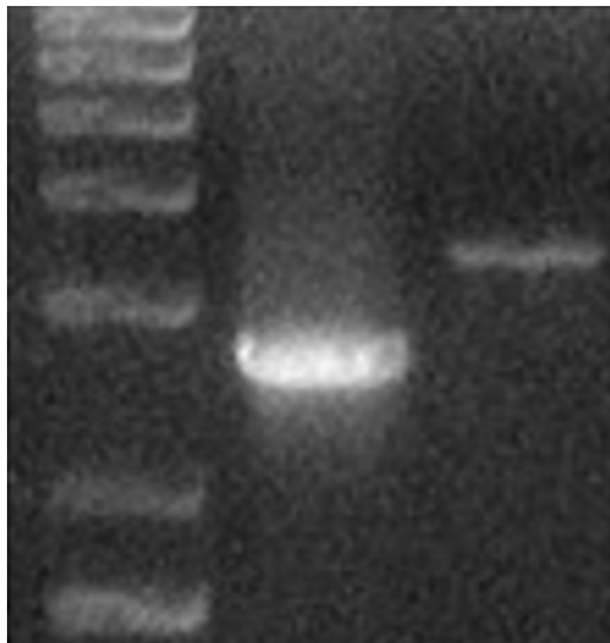

Figure 1. Agarose gel electrophoresis of the amplicons obtained with primers upstream and downstream of aae cloning sites (EXP5-AAE3) using as template DNA VT1169 (parental strain) (lane I) and USP29 *aae*⁻ mutant strain (lane II). Predicted fragment sizes: lane I, 3,551 bp; lane II 4,698 bp. MW: 1 Kb plus DNA ladder (Invitrogen Life Technologies).

Biofilm Formation

The parental strain (VT1169) showed greater ability to form biofilm than the *aae* null mutant (USP29). After 14 h of microaerophilic incubation, the *aae*⁻ mutant showed a 66.5% reduction in biofilm formation in relation to parental strain (Fig. 2C) ($p<0.001$, Student's t-test).

Adherence to SHA

Parental strain adherence to SHA averaged $20.06\pm2.99\times10^5$ CFU mL^{-1}, whereas only $1.55\pm0.29 \times 10^5$ CFU mL^{-1} of the *aae* null mutant strain were adherent. Thus, *aae*⁻ mutant's ability to adhere to SHA was significantly reduced when compared with the parental type ($p<0.05$, Student's t-test).

Discussion

In order to investigate the role of *aae* in oral cavity colonization by *A. actinomycetemcomitans*, an *aae* null mutant strain was constructed and its phenotype analyzed. This approach, used in several studies analyzing *A. actinomycetemcomitans* surface proteins, may provide insight of the interaction between multiple bacterial proteins on the cell outer membrane and host receptors (8,10).

Reduced hydrophobicity, decreased ability to bind to saliva-coated hydroxyapatite, and less biofilm formed than in the parental strain was observed. The collected data indicated that *aae* might also play a role in the interaction with salivary proteins coating the oral surfaces,

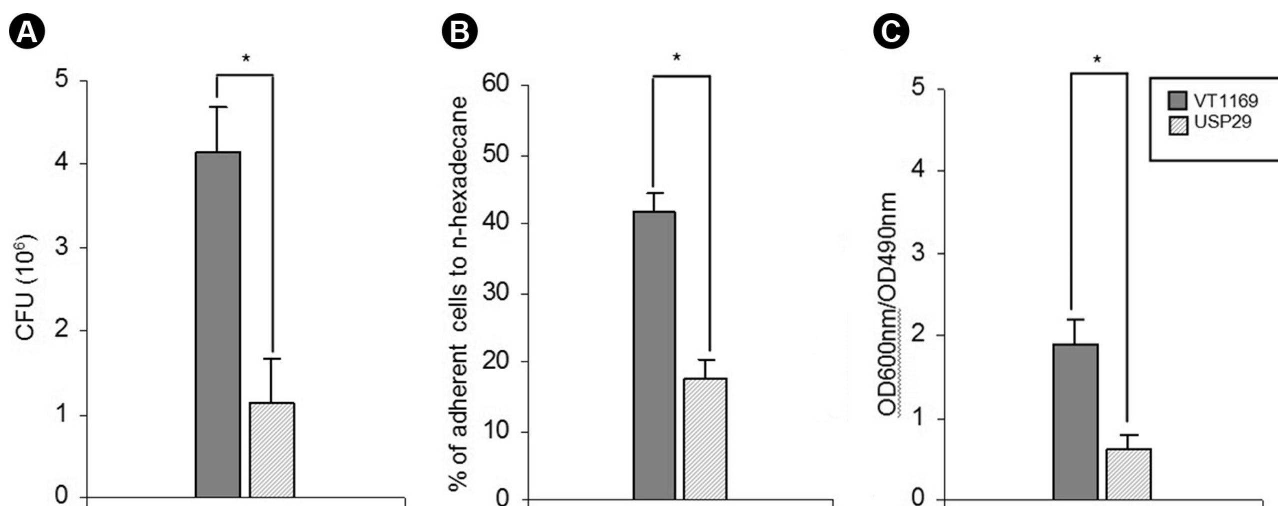

Figure 2. Phenotypic traits of *A. actinomycetemcomitans* parental (VT1169) and aae null mutant strains (USP29). Asterisk indicates statistically significant differences. A: Binding to KB cells (Student's t-test test, $p<0.01$). B: Percentage of bacteria adherent to n-hexadecane (Tukey's test, $p<0.0001$). C: Biofilm formation (Student's t-test, $p<0.001$).

increasing the repertoire of attachment mechanisms of *A. actinomycetemcomitans* in the oral cavity.

The ability of *A. actinomycetemcomitans* to bind to epithelial cells has previously been shown (8). In this study, the smooth variant (VT1169) showed higher level of binding to KB cells than the *aae* mutant strain (2-7 bacterial cells/KB cell vs. 1-2 bacterial cells/KB cell, respectively). Hence, the *aae*- strain exhibited reduced adhesion to KB cells, similar to values obtained in a previous study showing the aae role in adhesion to KB cells (8). Additionally to Aae, other adhesins like ApiA, are involved in *A. actinomycetemcomitans* adhesion to BECs (10,11). Residual levels of binding to KB cells found in the *aae*- mutant strain were thus expected.

A serotype b strain was chosen due to its correlation with aggressive disease, reaching high cell counts in the subgingival biofilm, possibly due to more efficient colonization mechanisms (19).

A smooth strain was chosen because the aggregative phenotype observed in rough variants limits calculation of the number of adherent cells to a substrate (6). The *aae* locus and the loci encoding other surface proteins, such as *api*A and *ema*A, reside outside the *tad* colonization island and, although smooth-surfaced *A. actinomycetemcomitans* variants have little or no fimbriation, they can adhere to epithelial cells and form biofilm (3,7).

Although both rough and smooth variants are able to produce biofilm in appropriate growth conditions, rough strains produced towers of microcolonies anchored by a small contact area, whereas smooth strains produced biofilm with a reduced height open architecture (7). Since a smooth variant was used, the biofilm studied here was independent from *flp*-1-mediated fimbriae.

Previous data have demonstrated that *aae* mutation in a rough strain did not seem to affect the capacity to attach to abiotic surfaces or to form biofilms (1). The present data using a smooth strain showed that biofilm formation was reduced in *aae*- null mutant. In smooth variants, its adhesion is mediated by specific non-fimbrial adhesins (9) and possibly other components, such as poly-N-acetyl-glucosamine (PGA) (20).

Bacterial binding results from a combination of affinity, electrostatic and hydrophobic interactions. Alterations in bacterial hydrophobic properties are expected when changes occur on cell surface protein composition (5,21). Thus, surface changes caused by *aae* interruption resulted in more hydrophilic cells. The decreased biofilm formation observed in the *aae* null mutant strain may indicate a role of *aae* in this process, either directly or due to changes in cell wall hydrophobic properties, influencing the cell-cell interaction.

The ability to bind to SHA is greater in rough strains of *A. actinomycetemcomitans* than in smooth variants (6) and addition of saliva to *A. actinomycetemcomitans* cells reduces adhesion to KB cells (3), indicating that these microorganism surface components interact with salivary proteins. The fimbriae are the main structure involved in biofilm formation in rough variants. However, the addition of polyclonal antiserum against a fimbrial peptide has only partially inhibited the adhesion of smooth variants (22), indicating a role of other surface components. It is currently known that fimbriae, lipopolysaccharide (LPS) and extracellular polymeric substance (EPS) play a role in the biofilm formation of *A. actinomycetemcomitans* and their expression is up regulated by iron limitation and anaerobiosis (23). Our research groups has previously shown that the transcription of *aae* is not up-regulated in an anaerobic environment (2), thus the present results obtained in microaerophilic atmosphere suggest that aae may be important in the adaptation to host environmental changes, such as increases in oxygen concentrations.

Resumo

O peridontopatógeno *Aggregatibacter actinomycetemcomitans* coloniza a cavidade oral aderindo e invadindo as células epiteliais e participando da formação de biofilme em superfícies duras. Aae, uma proteína autotransportadora está relacionada com a adesão bacteriana às células epiteliais. Devido às múltiplas funções desempenhadas por proteínas bacterianas autotransportadoras, este estudo teve como objetivo avaliar o papel de aae de *A. actinomycetemcomitans* tanto na capacidade de aderir à hidroxiapatita recoberta por saliva (SHA), quanto a de formar biofilme. Um mutante nulo aae foi construído. Suas propriedades hidrofóbicas, bem como a sia capacidade para aderir às células epiteliais, à SHA e para formar biofilme foram avaliadas e comparadas com a cepa –mãe, *A. Actinomycetemcomitans* VT1169. O mutante nulo aae apresentou redução de hidrofobicidade, assim como diminuição da adesão à SHA e na formação de biofilme, quando comparado à cepa parental. Estes dados sugerem que aae media a adesão de *A. Actinomycetemcomitans* às células epiteliais e pode também estar envolvida na formação de biofilme e na interação com proteínas salivares adsorvidas.

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